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FACTORS LIMITING CAPSULE SET, SEED SET AND REPRODUCTIVE SUCCESS IN *EUCALYPTUS GLOBULUS* SEED ORCHARDS

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Abstract

Low capsule set is a major factor limiting seed production in *Eucalyptus globulus* seed orchards. Trials were conducted in *E. globulus* seed orchards in Tasmania to identify the timing of capsule development and abortion, as well as the effect of pollination type, female tree, site, fertilisation, flower physical attributes, flower density, irrigation and weather events on capsule set and overall reproductive success. Controlled pollinations (CP), mass supplementary pollinations (MSP), open pollinations (OP) and un-pollinated controls (UP) were carried out at two orchard sites (Cambridge and Ridgley). Ramets of the same genotype differing in flower density and irrigation treatment were also compared for capsule set under MSP in three other experiments. No capsules were set in the UP, and capsule set was significantly lower following CP than OP and MSP. The genotypes at the lower altitude Cambridge site had a higher level of capsule set than the same genotypes pollinated at the Ridgley site. The major period of capsule abortion occurred between 20 and 80 days after pollination, coinciding with the period of capsule growth. There was a positive correlation between the number of fertilised ovules per aborted capsule and the length of time capsules were held on the tree. Flower physical attributes were found to correlate with reproductive success. Increased flower density had a negative impact on capsule set, as did irrigation. There was no clear evidence that weather events affected the rate of capsule abortion over the study period. Given that capsule abortion occurred during a period of fruit growth, that capsules with the lowest number of fertilised ovules aborted first and there is an impact from flower density and irrigation level, it is argued that fertilisation level and the level of resource competition are major factors determining capsule abortion.

Introduction

Eucalyptus globulus Labill. (Tasmanian blue gum) is a forest tree native to south-eastern Australia (Dutkowski and Potts 1999). It is the most widely planted hardwood species for pulpwood in temperate regions of the world (Potts et al. 2004), with 454,095 hectares of plantations in Australia alone (Parsons *et al.* 2006). While some clonal plantations have

been established in countries such as Chile, Portugal and Spain (Borralho *et al.* 1992; Griffin 2001; Lopez *et al.* 2002), most genetically improved plantations are still established from seedlings derived from seed orchards (Patterson *et al.* 2004a). Most seed orchard seed of *E. globulus* is from open-pollination, however, the development of single-visit (SVP, Williams *et al*. 1999) or one-stop (OSP, Harbard *et al.* 1999) pollination procedures for eucalypts based on style cutting, coupled with the large flower size of the species, has allowed the large-scale production of hand pollinated seed for deployment. This seed is produced from either one-stop controlled pollination (Harbard *et al.* 1999) or through mass supplementary pollination (MSP, Patterson *et al.* 2004a) and has the advantage that inbreeding depression arising from selfing is eliminated or significantly reduced (Hardner and Potts 1995) and specific full sib-families can be produced (Patterson et al. 2004a).

With the increased use of more expensive manual pollination for production of improved *E. globulus* seed, there is now a greater requirement to minimise reproductive losses during seed development. A major problem identified with hand-pollination of *E. globulus* flowers is that a significant proportion of hand-pollinated flowers do not set fruit (Rojas Vergara *et al.* 2001) which in eucalypts is a woody capsule. This would appear to be the case regardless of whether style cutting or traditional pollination procedures are used (Hardner and Potts 1995; Patterson et al. 2004a), and is often highly variable between female trees. For example in an Australian seed orchard, Patterson *et al.* (2004a) recorded that the proportion of flowers that form capsules that were retained on the tree until seed harvest (capsule set) following CP ranged from 10% to 90% between female trees. Studies of *E. globulus* seed orchards in Portugal have also shown capsule set to vary significantly between production seasons and specific trees within the orchard (Leal and Cotterill, 1997).

An understanding of the environmental and physiological processes influencing capsule set and abscission is therefore important in order to develop methodology to minimise reproductive losses following hand-pollination of *E. globulus* flowers. The present study aimed firstly to determine the time frame of morphological development of capsules after pollination, the timing of capsule abortion, and the fertilisation status of aborted capsules. Secondly, we aimed to study the effects of pollination technique, female tree, flower physical attributes, flower density, irrigation, and climatic events on capsule set and overall reproductive success.

Materials and methods

E. globulus trees used in his study were located in grafted seed orchards at (i) Cambridge, south-eastern Tasmania (42º48’27.23“S 147º25’58.48“E; alt: 40m; rainfall: 507mm) and (ii) Ridgley on the north-west coast of Tasmania (41º08’51.52“S 145º48’18.64”E; alt: 275m; rainfall 1200mm). The trees at Cambridge had been treated with the flower promoter paclobutrazol (Griffin *et al.,* 1993), were regularly topped and were irrigated whereas those at Ridgley had received no treatments.

A controlled-pollination trial involving 21 female trees of different genotypes was undertaken in 2004/05 at the Cambridge site. The 21 different females chosen for study had abundant flowers, and were unrelated first generation selections from the Furneaux, Western Otway and Strzelecki races of *E. globulus* as defined by Dutkowski and Potts (1999). Controlled pollination (CP) was undertaken to generate full-sib families using 4 flowers per pollen per tree and up to 100 pollens per female. All parental genotypes were unrelated to each other and CP pollinations involved crossing unrelated males and females. CP pollinations were undertaken by using the single visit pollination procedure outlined by Williams *et al.* (1999). Flowers on each tree were also (i) mass supplementary pollinated (MSP; 16 flowers per tree) with an operational mix of five different pollen parents following the procedure of Patterson *et*

al. (2004a) which involves style cutting but not emasculation or isolation, (ii) left for natural open-pollination (OP; 20-60 flowers per tree), and (iii) emasculated and isolated without style cutting or pollination to provide an un-pollinated control (UP; 16 flowers per tree). A small balloon placed over the floral rim was used to isolate the emasculated flowers in all cases. Approximately 12 months after pollination capsules were harvested and seeds extracted. Flower physical properties were measured for each genotype. After 4 weeks from pollination, aborted CP capsules were collected every 3-4 days and assessed for evidence of fertilisation following Pound *et al.* (2002). To monitor capsule growth, 5 capsules derived from MSP were harvested weekly for 29 weeks from two trees at Cambridge, and their fresh weight obtained. To investigate possible links between climatic factors and timing of capsule abortion, data for rainfall, wind speed, relative humidity and temperature were matched with the CP abortion data.

Crossing was also conducted at Cambridge in 2005/06 on 19 female trees of different genotypes from the same three races, many of which were the same as used in the previous year. In this season, fresh (4 genotypes) and low viability stored pollen (21 genotypes) was used for CP crossing. Pollinations were also conducted in Ridgley in 2005/06 on 6 genotypes that had been crossed in the previous year at Cambridge. CP, MSP, OP and UP were performed on 16 flowers of each tree. A fresh mix of 5 pollens was used for MSP and low viability stored pollen was used for CP.

To test the influence of flower density; two ramets of similar canopy size, subject to the same pollination type (MSP or OP) and location within the Cambridge orchard but differing in flower density were selected from each of 12 genotypes. Capsule set was measured in 2004/05 by placing a litter trap under each tree and counting the number of opercula shed and flowers aborted. To measure the influence of irrigation deficit; in 2005/06 two ramets of similar size, same pollination type (MSP or OP), location within the orchard and flower density were selected for each of 7 genotypes. The irrigation was switched off for one ramet, while the other received the normal level (approximately 42L per week). In 2006/07 three ramets were selected for each of 7 genotypes. These trees were different to those used in the previous year. Irrigation was switched off for one ramet, the other received half on only one side of the tree with sides were swapped every two weeks and the other ramet received full irrigation. The average rainfall at Cambridge from the period from pollination to capsule set is 177.3mm. Five shoots on each tree were measured and labelled at pollination and were re-measured at the end of the capsule set period, to determine vegetative growth.

To test the influence of flower physical and physiological parameters on reproductive success, genotypes were chosen representing a range of reproductive success levels on the basis of the 2004/05 and 2005/06 Cambridge seed per flower data. Six genotypes were used, two representing each of the Furneaux, Strzelecki and Western Otway races, and within each race one of the genotypes selected was a good performer and the other poor, in terms of reproductive success. Trees were pollinated with 6 different pollen genotypes (two from each race), 7 flowers were pollinated with each, and thus 42 flowers were pollinated on each tree. 6 flowers on each tree, one from each cross, were sampled on days 2, 7, 14, 28 and 42 after pollination. At approximately 100 days after pollination, when the seeds were set, the remaining capsules, along with 10 open pollinated capsules from each tree were harvested, dried and seed was extracted. Samples were assessed for pollen tube numbers within the style, ovule penetration (Martin, 1959) and fertilisation (Pound *et al*. 2002). Flower physical measurements were made for all genotypes.

Data for the flower density and irrigation trials were analysed using a paired t-test. SAS version 9.1 (SAS Institute Inc 2003) was used for all other statistical analysis. The significance of pollination type was tested by a one-way analysis of variance using PROC GLM. PROC REG was used to test the fertilisation of aborted capsule data and flower physical and physiological properties verses reproductive success data. A one-way analysis

of variance using PROC MIXED was used to determine the female effect on reproductive success, and the consistency of reproductive success across seasons was determined by Kendall's tau-b correlations.

Results

Within the Cambridge site in 2004/05 significant differences in capsule set were recorded between the three pollination treatments at harvest time ($F_{2, 60} = 7.35$; $P<0.001$). No capsules set for UP, while CP (73.2%) set a significantly lower number of capsules than OP (87.9%) and MSP (90.2%) treatments. Viable seed set per capsule also differed significantly between pollination types at Cambridge in 2004/05 ($F_{2, 60} = 5.33$; $P<0.008$). CP (45.3 seeds per capsule) was significantly higher than both MSP (30.8 seeds) and OP (21.3 seeds). The differences in overall reproductive success were, however, not significant ($P>0.05$): CP (33.2 seeds per flower), OP (18.8) and MSP (27.8).

Only one out of 1237 harvested CP capsules contained zero viable seeds. In 2005/06, seed set at Cambridge for CP averaged 18.8 seeds per capsule, and 27.5% of capsules set contained zero viable seeds. In 2005/06 seed set at Ridgley for CP averaged only 0.7 seeds per capsule and 40% of the CP capsules set contained zero viable seeds.

Starting from the first sample date, 7 days after pollination, average fresh capsule weight in Cambridge 2004/05 increased linearly from 1.1g to 3.1g by day 70, after which there was effectively no weight gain, at least up until the sampling ceased at day 147 for the two trees studied. Capsule abortion for CP at Cambridge and MSP, and UP at both Cambridge and Ridgley occurred primarily between 20 and 80 days after pollination or isolation treatment (MSP and UP are shown in Figure 1). All UP flowers except one aborted by day 80. A significant linear relationship ($Y=0.18X - 0.71$, $r^2=0.75$; $P<0.001$) was identified at Cambridge between the number of fertilised ovules within aborted CP capsules and the time after pollination that capsules aborted. Aborted capsules that were held on the tree for the longest had the highest number of fertilised ovules. No obvious relationship occurred between capsule abortion and weather observations recorded over the 3-4 day time interval between collection times.

When the six common female genotypes were compared for the success of MSP between seasons and sites, capsule set at Cambridge 2004/05 (91.7%) was significantly ($F_{1,11} = 5.48$; $P<0.05$) higher than that at Ridgley 2005/06 (58.2%). Cambridge MSP (36.5) and OP (27.9) seed per capsule were higher than that at Ridgley (22.9) and (15.1), although the difference was not significant ($F_{1, 11} = 1.59$; $P>0.05$).

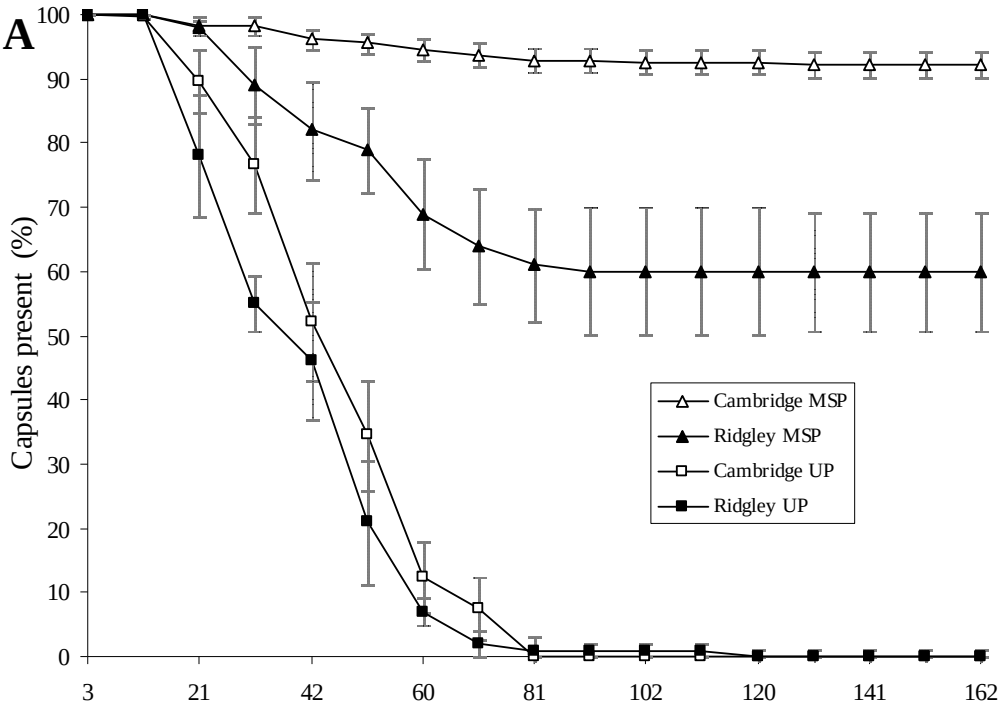

Fig. 1 The mean (± s.e.) percentage capsules retained with time for pollinated flowers (MSP) and un-pollinated flowers (UP) at Cambridge 2004/05 and Ridgley 2005/06. The data is averaged over 21 and 6 trees of different genotype for the Cambridge and Ridgley orchards respectively. In each case, high viability pollen mixes (collected in the same season) used in commercial operations were used for MSP crossing.

When genotypes were placed into pairs of high and low flower density trees, the level of capsule set was significantly (paired t_{11} = 3.15; P<0.001) higher in the low flower density trees. Low flower density trees had a capsule set of 81.6 (±6)% and high flower density trees a capsule set of 67.8 (±6)%.

When irrigation deficit treatments were initially performed on paired genotypes subject to MSP and OP in 2005/06, trees that received no irrigation had slightly lower level of capsule set 51.3 (±8)% than trees that received full irrigation 61.6 (±6)%, but the difference was not significant. In the following year when a partial root zone drying treatment was added to the experiment, the fully irrigated treatment had the lowest level of capsule set 53.7 (±9)% followed by the partial root zone drying (PRD) treatment 67.8 (±8.5)% then by the non-irrigated treatment 74.6 (±7.5)%. Both the dry (paired t_6 = 2.36 P<0.05) and PRD treatments (paired t_6 = 2.55; P<0.05) had significantly greater capsule set than the fully irrigated treatment. Shoots measured for vegetative growth for non-irrigated trees had an average relative increase of 2.28 (±0.37) times the initial shoot size, the average relative increase for the partial root zone drying treatment was 2.95 (±0.23), and for the full irrigation treatment was 3.26 (±0.37). The relative increase in shoot length of fully irrigated trees was significantly different from the dry treatment (paired t_6 = 2.36; P<0.05).

When site, season and irrigation are kept constant and pollen collected in the same season is used, the success of cross-pollination in terms of seed per flower is predominantly determined by the female genotype. This was evident for the 2004/05 season of CP crossing at the Cambridge site. When 21 trees were crossed as females with 14 different, unrelated pollens, the female effect ($F_{20, 242}$ = 2228; P<0.01) was larger than the pollen effect ($F_{13, 242}$ = 229; P<0.05) and there was no significant interaction effect (F_{242} = 63 P<0.1). Where common genotypes were crossed in different seasons at Cambridge, female genotype differences in seed per flower crossed was found to correlate across seasons (e.g.

Kendall's tau-b for 2004/05 vs 2005/06 CP; r_4=1.0; P<0.05; 2004/05 and 2006/07 r_6=0.87; P<0.05).

Subsequent morphological and anatomical studies in 2006/07 using pairs of genotypes from each of the three races which showed large differences in the number of seed obtained per flower crossed in 2004/05 suggested that variation in reproductive success between trees used as females could be related to both floral physical and physiological differences. The female differences in reproductive success in 2004/05 were associated with physical properties of the flower including: capsule width (mm; Y=11X-165; r^2 = 0.9; P<0.01), style length (mm; Y=34X-194; r^2 = 0.89; P<0.01), total number of ovules (Y=0.4X-50; r^2 = 0.84; P<0.01) and area of conductive tissue at 1mm in the style (mm^2; Y=318X-286; r^2 = 0.76; P<0.05). However, when specific differences in reproductive success were not explained by physical differences in the flowers, they appeared to be related to physiological properties of the style as there was also a significant overall relationship between reproductive success and the proportion of germinating pollen tubes which reached the bottom of the style (Y=401X-115; r^2 = 0.84; P<0.01).

Discussion

Despite the large difference in the level of capsule abortion between sites, seasons and pollination treatments, the timing of abortion was consistent. Capsule abortion appeared mainly confined to a period after pollination (20 and 80 days), coinciding with the period of capsule growth (0 to 70 days). As was found in the present study, the major period of abortion in horticultural trees occurs during the initial rapid growth phase, whereby up to 80% of the young fruit losses occur within 60 days of anthesis and this is generally related to seed development (Sedgley and Griffin, 1989).

The success of crossing, whether CP or MSP, at Cambridge in 2004/05 was amongst the highest yet reported in *E. globulus*. The lower level of CP capsule set compared with MSP and OP may simply reflect the additional handling and damage to the flower associated with this treatment (e.g. emasculation, style cutting and isolation). Wounding can directly stimulate abscission of plant structures, and provides possible entry points for pathogens, stimulating defence responses that can lead to abortion (Taylor and Whitelaw 2001). However, this deleterious effect is not reflected in the high seed set per capsule observed in CP. The reduced seed set per capsule in OP may reflect either pollen limitation due to dependence on natural pollination (Hingston and Potts 1998), the enhanced capacity for seed set often observed following style cutting (Harbard *et al.* 1999) and/or the abortion of selfed fertilised ovules produced under open pollination (Hardner and Potts 1995). The later could also explain the reduced seed set in MSP compared to CP as MSP flowers are exposed to small amounts of pollination by self pollen as flowers are not emasculated nor isolated (Patterson *et al.* 2004b).

The comparison of the MSP at Cambridge and Ridgley, as well as OP and comparable CP crosses, suggests that the Ridgley site is inherently poorer for both capsule set and seed set per capsule. General site effects on reproductive parameters such as capsule and seed set have been reported in other eucalypt studies (Harbard *et al.* 1999). While differences in pollinator efficiency or abundance could account for the difference in capsule set and seeds per capsule between OP's at each site, this does not explain the reduced success of the MSP treatment at Ridgley. This effect is unlikely to be a random seasonal effect as poor capsule and seed set at this site has previously been reported in CP crosses undertaken in other seasons (McGowen 2007). Extreme weather events such as frosts, wind and unusually high temperatures have been shown to promote fruit abortion (Stephenson 1981) and may account for the site difference observed, however, such weather events did not

appear to be a significant factor in explaining the small abortion of capsules at the low altitude Cambridge site in 2004/05.

Within the CP crosses the timing of capsule abortion appeared to relate to the number of fertilised ovules with the later aborted capsules having greater numbers of fertilised ovules than those that aborted earlier. Based on comparing the average fertilisation values of the aborted capsules with the CP seed set values and the number of ovules that are reported to be available for reproduction (Pound *et al.* 2002), most abortion was associated with poor fertilisation of ovules. The temporal pattern of capsule abortion was consistent with the 'bet hedging' hypothesis; whereby developing fruits with the least reproductive potential abort first, followed by those with a slightly higher reproductive potential up to a point where the available resources are sufficient to retain the remaining fruit on the tree. Capsules with weaker sink strength, resulting from a lower number of fertilised ovules, may not have the competitive ability to draw resources required to grow and as a result abort (Wesselingh 2007). Resource competition has also been shown to exist between ovules within capsules in *Eucalyptus regnans* (Griffin *et al.* 1987). This theory may also explain why the larger flowers having a higher reproductive success, as the presence of larger capsules with more ovules and larger styles with more conductive tissue, may increase the capsules sink strength, increasing the chances of capsule set. When the differences in capsule physical properties could not explain the difference in reproductive success, in the cases studied it appeared to be explained by the ability of the style to mediate pollen tube growth. Thus, genotypes that were letting a higher proportion of pollen tubes through to the base of the style had higher fertilisation, increasing the capsules sink strength. Increased capsule set with the non-irrigated treatments is also consistent with the resource competition theory as there was less vegetative growth for the non-irrigated treatments. According to Sedgley and Griffin (1989) fruit set in fruit trees may be increased by controlling irrigation so as to reduce vegetative shoot growth immediately after flowering.

Only one capsule, or less than 0.1% of the total CP harvested at Cambridge in 2004/05, contained no viable seeds, which was consistent with the observation of Griffin *et al.* (1987) that parthenocarpy has not been reported for *Eucalyptus*. However, the number of seedless capsules increased to 28% and 40% in Cambridge and Ridgley respectively in 2005/06 when less viable pollen was used. A higher incidence of capsule abortion and lower seed set was also recorded for CP flowers in 2005/06, suggesting that competition for resources by developing capsules was less than in the previous year. The abortion of all UP capsules suggests that the development of seedless capsules may not be due to vegetative parthenocarpy (Sedgley and Griffin 1989). Instead, it appeared to be due to either stimulative parthenocarpy (presence of pollen tubes), or stenospermocarpy (abortion of fertilised ovules) (Sedgley and Griffin 1989). Fruits with lower seed numbers are tolerated by plants when resources are plentiful and/or when the general level of pollination is low (Wesselingh 2007).

In conclusion, most capsule abortion in *E. globulus* occurs during the period of capsule growth, over the first 80 days and the main driver appears to be the level of ovule fertilisation of the flower. Fertilisation is not only influenced by pollination but also some physical and physiological properties of the flower. The timing of capsule abortion is consistent with resource competition such that poorly fertilised capsules are those that abort first. However, the extent to which poorly fertilised capsules with low seed development are retained may vary depending on levels of resource competition, as well as the competitive environment and ability of the capsules. Under conditions of low competition even capsules with no viable seed may develop to maturity.

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